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CONTROVERSIAL ROLES OF MAST CELLS IN RHEUMATOID ARTHRITIS

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Rivellese F, Suurmond J, Habets K, Dorjée AL, Ramamoorthi N, Townsend MJ, de Paulis A, Marone G, Huizinga TW, Pitzalis C, Toes RE.

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II. Reply to: Serum levels of tryptase suggest that mast cells might play an anti-inflammatory functions in Rheumatoid Arthritis

Rivellese F, de Paulis A, Marone G, Pitzalis C, Toes RE.

Arthritis Rheumatol. 2016 Mar; 68(3):769-70.

III. IgE and IL-33-mediated triggering of human basophils inhibits TLR4-induced monocyte activation

Rivellese F, Suurmond J, de Paulis A, Marone G, Huizinga TW, Toes RE.

Eur J Immunol. 2014 Oct; 44(10):3045-55.

IV. Toll-like receptor triggering augments activation of human mast cells by anti-citrullinated protein antibodies.

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V. Synovial mast cells correlate with synovial inflammation and cellular infiltration and are functionally associated with ectopic lymphoid structures in patients with early rheumatoid arthritis.

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Manuscript in preparation

VI. Synovial mast cells predict of radiographic progression in patients with early rheumatoid arthritis

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Manuscript in preparation

ABSTRACT

Background

Mast cells are tissue-resident cells of the innate immunity implicated in the pathogenesis of many autoimmune diseases, including rheumatoid arthritis (RA). In fact, they are present in synovia and their activation has been linked to the inflammatory responses driving the development of RA. However, their exact contribution to the development of RA is still unclear. In particular, their interactions with other immune cells at synovial levels and their correlation to disease outcomes have never been investigated in a systematic fashion.

Objective

Aim of this PhD project was to elucidate the role of mast cells in rheumatoid arthritis, by studying their cellular interaction at synovial levels, their influence on the activation of immune cells and, finally, their correlation with disease outcomes.

Results

In the synovia of RA patients, mast cells correlated with the degree of local inflammations and cellular infiltration. In particular, higher numbers of mast cells were associated with the presence of lymphoid aggregates, and mast cells were localized in close proximity of cellular aggregates of B and T cells. In vitro, mast cells were able to modulate the responses of immune cells, suppressing the pro-inflammatory activation of monocytes and supporting B cells survival, differentiation and antibody production. In patients with early RA, high numbers of synovial mast cells were associated with antibody positive disease and with systemic inflammatory markers. At the 12 months follow-up, baseline mast cells represented an independent predictor of radiographic progression.

Conclusions

Mast cells, as part of the inflammatory infiltrate in the synovia of RA patients, are able to induce both pro- and anti-inflammatory immune responses, which might explain the conflicting results obtained so far with experimental models not taking into account their tunable immunomodulatory properties. The ex vivo analysis performed in the

synovia of early RA patients indicate that mast cell presence can be used to further dissect the disease heterogeneity, as mast cells clustered with antibody positivity and with a higher degree of local and system inflammation. Interestingly, independently from these associations, mast cell presence at baseline represented a predictor of radiographic progression at 1 year. Overall, our data suggest that mast cells play a multifaceted role in the pathogenesis of RA, warranting additional investigations to further assess their involvement in disease development, progression and response to therapy.

INTRODUCTION

Mast cells as effector cells of innate and adaptive immunity

Mast cells (MCs) are granulated tissue-resident cells of hematopoietic lineage [1]. Deriving from specific hematopoietic precursors, they circulate as immature cells and migrate into vascularized tissues, where they complete their differentiation and reside in the proximity of blood vessels. As they are present in all anatomic sites exposed to the external environment, they represent, together with dendritic cells, the first cells of the immune system to interact with environmental antigens, pathogens and toxins. Therefore, they can be considered "sentinels" of the immune system [2], localized on the frontline, and ready to fight in response to infections and other stimuli. In fact, mast cells express many receptors (i.e. complement receptors, Fcγ, and Toll-like receptors), and are able to sense danger signals and react by quickly releasing a wide range of mediators, both pre-formed (e.g. histamine, etc) and newly produced (cytokines and chemokines).

Even though these innate immunity functions partially overlap with other immune cells (e.g. neutrophils, macrophages, dendritic cells), evidences

from animal models demonstrated that the absence of mast cells significantly impairs the ability to respond to bacterial infections [3,4].

In line with their importance in the fight against pathogens, it is also worth noticing that mast cells are highly conserved throughout evolution [5], as MC-like cells, containing histamine and heparin, have been described in very simple invertebrates [6]. In higher species, with the evolution of adaptive immunity, mast cells acquire the expression of the high affinity receptors for IgE (FceRI) [7]. Triggering of the IgE bound to the FceRI induces mast cells to degranulate, with the quick release of pre-formed mediators. Because of this property, together with circulating basophils, mast cells are mainly known as effector cells of IgE-mediated (Th2) responses, an arm of the adaptive immune system develop to fight multicellular pathogens, such as helminths [8], and in recent years recognized as the mechanism at the basis of hypersensitivity reactions [9].

Mast cells and the modulation of adaptive immune responses

In addition to their functions as effector cells, recent evidences suggest that mast cells are able to modulate the adaptive immunity, therefore representing an important link between innate and adaptive immunity [10].

For example, mast cells express MHC class II and have been shown to induce antigen-specific T cell activation [11-13]. Vice versa, mediators derived from cells of the adaptive immunity can influence mast cell activation, as shown for example by the ability of IgG to activate mast cells through the FcgammaR [14,15]. In the crosstalk between mast cells and adaptive immunity, the protective role of mast cells in the response against pathogens, i.e. their ability to orchestrate Type 2 responses against helminths [11,16], can become harmful when the immune response is dysregulated, and mast cells can potentially contribute to the deleterious responses leading to allergy and autoimmunity [5].

A great deal of evidences indicate that mast cells have deleterious effects in these contexts, given their ability to release mediators with clear pro-inflammatory effects, such as histamine, and several prostaglandins and cytokines.

Albeit mostly known for their pro-inflammatory effects, many of these mediators have been also shown to have opposite effects.

As an example, histamine is well-known for its pro-inflammatory functions (e.g. vasodilation, increased vascular permeability, bronchospasm etc). However, we have shown that basophil-derived histamine is able to shown

to suppress the pro-inflammatory activation of monocytes [17], thereby contributing to the down-regulation of immune responses.

Although apparently contra intuitive, it is not surprising that the same cells, mechanisms and mediators responsible for the early inflammatory responses (e.g. histamine-induced vasodilation) are also able to suppress inflammation (e.g. the above cited histamine-induced immune modulation). In fact, resolution is a process starting early in the course of inflammation in order to restore homeostasis, prevent tissue damage and facilitate wound repair.

In line with this concept, mast cell have been shown to produce mediators known for their immune regulatory effects toward the resolution of inflammation [18], such as IL-10, and mast cell-derived IL-10 has been shown to have protective effects in vivo [19-21].

Overall, mast cells should be considered more than effector cells, as they are able to orchestrate the adaptive immune responses, acting as immunomodulatory/ regulatory cells that can be finely tuned by multiple stimuli [22,23].

Mast cells in rheumatoid arthritis

As immune cells present the synovial membrane, mast cells have been implicated in the pathogenesis of many rheumatic diseases, including Rheumatoid Arthritis (RA) [24]. The synovial membrane is the primary inflammatory site of RA and its inflammation – i.e. synovitis - is mainly characterised by thickening of the synovial layer, with cellular hyperplasia and infiltration of immune cells [25]. Mast cells are among the immune cells found in the inflamed synovia of RA patients. They are known to be present as resident cells in healthy synovia [26], and an increase in their numbers has been shown to accompany the cellular hyperplasia in RA [27-29]. Starting from this observation, many attempts have been made to further elucidate their involvement in the disease process leading to RA. Robust laboratory evidences support the hypothesis of a pro-inflammatory (pro-arthritogenic) role of mast cells in the pathogenesis of RA, and the pathways leading to mast cell activation in the context of rheumatic diseases and their effector functions have recently been extensively reviewed [30]. However, mast cell activation in inflamed tissues is sometimes considered as a mere consequence of tissue inflammation, rather than playing a specific role in disease pathogenesis. The functional contribution of mast cells to RA and

other autoimmune diseases is difficult to assess in patients, because of the lack of specific mast cell inhibitors for clinical studies. Therefore, the study of their functional relevance relies on the use of animal models, that in most cases do not fully reproduce the human disease, only representing one component of the immune response [31]. Therefore, it is not surprising that different animal models generated inconclusive results, failing to confirm specific role for mast cells, so that many of their functions have been labelled as redundant. More recently, the inconsistency of the results in vivo has been attributed to the experimental flaws of the techniques to obtain mast cell depletion [32]. In fact, early experiments used models of mast cell depletion based on mutations affecting c- kit (Stem Cell Factor receptor) structure or expression and that consequently exhibit a profound MC deficiency together with a variety of other phenotypic abnormalities. The promising results obtained in such models have been subsequently challenged by using new 'KIT-independent' models of MC deficiency, so that, in many contexts, the involvement of mast cells have been now labelled as redundant [33].

A recent publication further addressed the complexity of the animal models to study the involvement of mast cells in RA, finally shedding some light

on the inconsistent results obtained so far [34]. Schubert and colleagues studied MC functions using both antibody-induced and antigen-induced experimental arthritis in Mcpt5-Cre;iDTR mice as a model of inducible MC-deficiency that is independent of Kit mutations and reflects a normal immune system despite the absence of MCs. Consistently with previous findings [35] MC role was found to be redundant in the K/BxN serum-induced joint inflammation. However, in this model, joint inflammation is induced passively by the injection of an arthritogenic serum, therefore only assessing the effector functions of the immune system. This is suggesting that mast cells are dispensable for this type of response, which is only a part of the complex immune response leading to the development of RA. On the contrary, mast cell depletion significantly reduced the disease severity in a model of collagen-induced arthritis, based on an active immunization with an antigen (collagen), more accurately mimicking the early phases of RA, in which mast cells could contribute to the breach of tolerance against self-antigens. The conclusion from this study is that mast cells could play a role in the early immune response in the course of arthritis, while their functions seem to be redundant in the later effector phases.

Albeit fascinating, the transfer of these results to human disease is complex, especially because animal models rely on genetically identical mouse strains, clearly not taking into account the heterogeneity of the human diseases, which is particularly true for RA, well recognized as a highly heterogeneous disease, with enormous differences between patients in terms of pathogenesis [36], disease progression and response to therapy[25,37]. In this context, the previously discussed evidences of the anti-inflammatory and immunomodulatory roles of mast cells could offer an alternative explanation for the conflicting results obtained in different animal models.

In particular, it is intriguing to speculate that mast cells, as tunable effector cells, have various functions in different patient subsets, and, possibly, in different disease stages, adding more complexity to the heterogeneity of RA.

The only way to confirm this hypothesis would be to study the presence of mast cell directly at synovial level, which has been challenging in the past, with a few studies relying on synovial tissue obtained by arthroscopy or during surgery in patients with long-standing disease [38,39]. However, the recent technological advancements of ultrasound-guided synovial biopsies allowed to obtain synovial tissue from patients with a minimally invasive

procedure [40]. Therefore, synovial histology has been proposed as a tool to dissect the heterogeneity of RA, and the study of synovial histopathology in early patients has led the stratification of patients in subgroups, according to histological patterns (pathotypes), that are emerging as potential taxonomic classifiers of disease phenotypes [41]. In particular, synovial histopathology, depending on predominant type of infiltrating cells, has been defined as fibroid, myeloid and lymphoid. The latter is characterized as lymphoid follicle-like structures ranging from T and B cell aggregates to highly organized structures comprising follicular DC (fDC) networks reminiscent of germinal centres (GCs). These structures are present in up to 40% of RA patients [41,42] and have been associated with severe disease, T cell priming, and autoantibody production [43-45]. Particularly interesting is the evidence that these ectopic lymphoid structures (ELS) support the local ongoing production of class-switched autoantibodies [45], including anti-citrullinated protein/peptide antibodies (ACPA), which are highly specific markers of RA, predict a poor prognosis, and have been suggested to be pathogenic.

Although mast cell presence in synovia has been known for a long time, it is not clear if their presence is associated with any of the pathotypes and,

ultimately, if the analysis of synovial mast cells could contribute to a better definition of the synovial histopathology.

Aim of this PhD project was to elucidate the role of mast cells in rheumatoid arthritis, by studying their cellular interaction at synovial levels, their influence on the activation of immune cells and, finally, their correlation with disease outcomes.

MATHERIALS AND METHODS

Peripheral blood derived mast cells.

Buffy coat cells were obtained from the peripheral blood of healthy volunteers, with samples collected from a blood bank. $CD34^+$ hematopoietic stem cells were isolated from peripheral blood mononuclear cells (PBMCs) using CD34 microbeads (Miltenyi Biotec). Isolated $CD34^+$ stem cells were differentiated into mast cells using a previously described method [46]. After 6–8 weeks, the purity of the mast cells was determined by flow cytometry analyses for the expression of CD117 (c-Kit), Ig E-class–binding Fc receptor type I (FcERI), and CD203c; purity ranged from 90% to 99%.

Mast cell activation

Plate bound IgG were used as a model to study Fcγ-receptor-mediated-activation of mast cells [47,48]. Briefly, culture plates (flat-bottom 96-wells or 48-wells) were coated with 100 μg/mL of purified human IgG (Jackson Immunoresearch) in PBS for 1,5 hours at 37 °C and washed 2x with PBS Mast cells were cultured at a concentration of $1x10^6$/mL in RPMI-1640

medium containing 10% fetal calf serum (FCS), glutamine, penicillin, streptomycin (Invitrogen) + 100 ng/mL of SCF (Tebu-bio), without or with 100 ng/mL of recombinant human IL-33 (PeproTech). After 24h, cells were harvested, supernatants collected and stored at -20°C and cells used for flow cytometry analyses.

IgG-ACPA mediated mast cell activation

CCP2 peptides were obtained from Dr. Drijfhout, Department of IHB, LUMC, The Netherlands. Nunc Maxisorp plates (VWR) were coated with CCP2 peptide or arginine control peptide and incubated at 37 degrees for 1 hour with serum of ACPA-positive RA patients, diluted 50x in PBS/0,1%BSA. After washing, mast cells were added to the wells, and cultured in the presence or absence of 100 ng/mL of recombinant human IL-33. After 24h, cells were harvested, supernatants collected and stored at -20°C.

Monocyte isolation and stimulation with Mast cell supernatants

$CD14^+$ monocytes were isolated from buffy coats PBMCs using magnetic-labeled anti-CD14 beads (Miltenyi Biotec), according to the manufacturer's

instructions. Isolated monocytes (purity > 95%) were cultured in the same medium used for mast cells.

Monocytes were incubated with mast cell supernatants (diluted 1:4 in medium) or control media and stimulated with lipopolysaccharides (LPS) from *Salmonella typhosa* (Sigma-Aldrich) at a concentration of 5 ng/mL. After overnight (18h) incubation, cells were harvested, supernatants collected and stored at -20°C for further analysis. Cells were used for flow cytometry analyses. For blocking experiments, supernatants of activated mast cells were pre-incubated with anti-IL-10 antibody or matching isotype control Rat IgG2a (BDBiosciences) at 10 μg/ for 30 min at 37°C in 5% CO2 atmosphere, prior to addition to the monocytes. For inhibition of histamine, monocytes were pre-incubated for 30 min at 37°C with histamine receptor 2 antagonist ranitidine at 10^{-4} M (Sigma) [17].

B-cell isolation

Human B cells were immunomagnetically selected from tonsils obtained following routine tonsillectomy. Briefly, tonsils were washed with chilled (4°C) MACS buffer (phosphate buffered saline (PBS), 0.5% FCS and 2 mM EDTA), minced and gently mashed through a cell strainer. Cells were washed, centrifuged and B cells were isolated using anti-IgD microbeads

(Miltenyi Biotec), according to the manufacturer's instructions. The purity of IgD+ unswitched B cell isolation was checked by fluorescence activated cell sorting.

Co-culture of B cells and Mast cells

IgD+ B cells and mast cells were resuspended at a concentration of $1x10^6$/mL in IMDM-1640 medium containing 10% fetal calf serum (FCS), glutamine, penicillin, streptomycin (Invitrogen). In cell–cell contact experiments, B cells (1×10 6 cells/300 μl/ well) were cultured together with mast cells at a ratio of 1:6 (1x10/ 50 μl/ well) In transwell experiments, microporous PET cell inserts (3 μm pore) were employed, with mast cells in the upper well and B cells on the base of the well. B cells were activated with 1ug/mL of CPG. After 6 days, cells were harvested, supernatants collected and stored at -20°C for further analysis and cells were resuspended for FACS analysis. In parallel experiments, B cells were labelled with CFSE (Biolegend) a 5 μM, according to the manufacturer's protocol.

Patient samples

Serum samples were obtained from RA patients, with the presence of total IgG-ACPA tested by routine diagnostic ELISA. Synovial tissue for

immunofluorescence analysis was obtained from 3 patients (aged 59, 70 and 76) with ACPA+ established RA patients who had undergone surgery. For mRNA sequencing, mRNA was extracted from the synovial tissues obtained from ultrasound guided biopsies of patients with early (< 12 months) active RA naïve for DMARDs therapy (n=40), enrolled in the Pathobiology of Early Arthritis Cohort (PEAC) cohort http://http://www.peac-mrc.mds.qmul.ac.uk [41] of the Centre for Experimental Medicine and Rheumatology of Queen Mary University (London). Additionally, the presence of mast cells was analysed by immunohistochemistry in a total of 90 patients from the PEAC cohort. The PEAC is a prospective, observational study in patients with early symptomatic arthritis (3-12months durations), in which patients were categorized, monitored and treated according to best practice. X rays at 0 and 12 months were scored according to the Van der Heijde-modified Sharp method [49] and radiographic progression was defined as an increase in the Van der Heijde-modified Sharp score by more than 1 points over 1 year. All patients selected for our analyses fulfilled the 1987 revised American College of Rheumatology criteria for RA [50]. Written informed consent was

obtained from the patients, and the study was approved by local human ethics committees.

Immunofluorescence

Samples were fixed by 4% (wt/vol) formaldehyde (Merck) in PBS and stored in 70% (vol/vol) ethanol. Tissues were embedded in paraffin, sectioned at 4 μm. Slides were deparaffinised with xylene (Merck), endogenous peroxidase activity was blocked with 1% hydrogen peroxide (Merck) in methanol for 10 min. After antigen retrieval with a Tris/EDTA solution (pH9; DAKO) for 30 min at 96 °C, slides were stained with primary antibodies (tryptase, CD117, CD14, CD3, CD20) or corresponding isotypes (Dako). Detection was performed using the appropriate secondary antibodies (Invitrogen). All slides were mounted with Vectashield Hard Set mounting medium with DAPI (Vector Laboratories). Slides were stored in the dark at 4ºC before visualisation using a Zeiss Axio Scope A1 and AxioVision 4.9.1 or a *microscope name* and cellSens software (Olympus).

Immunohistochemistry analysis of synovial tissue

Sections of paraffin embedded synovial tissue obtained by ultrasound-guided synovial biopsy from DMARD-naïve patients with early (<12

months) RA (n=90) were stained with H&E and underwent semi-quantitative (SQ) scoring (0-9) to determine the degree of synovitis, according to a previously validated method [51]. Sequentially cut sections were stained by IHC for CD117+ MCs and immune cells (CD20+ B cells, CD3+ T cells, CD68+ macrophages and CD138+ plasma cells). Upon SQ scoring (0-4), sections were stratified into synovial pathotypes according to the degree of immune cell infiltration: (i) Lymphoid- grade 2/3 B cell aggregates, CD20≥ 2 and/or CD138>2 ii) Myeloid- CD68 SL≥ 2, CD20≤1 and/or CD3≥1, CD138≤2 and iii) Fibroid- CD68 SL<2 and CD3, CD20, CD138<1).

Flow cytometry

For flow cytometry staining, cells were incubated with fluorochrome-conjugated antibodies diluted in PBS 0.5% BSA at 4 ºC for 30 min. To exclude dead cells, just prior to flow cytometric acquisition, 0.2 uM DAPI (Invitrogen) was added. Flow cytometric acquisition was performed on LSR-II (BD). Analysis was performed using FACS Diva (BD) and FlowJo software (Tree Star Inc.).

Measurement of cytokines and immunoglobulins

Quantitative immunoassays in mast cell culture supernatants were performed using the 42-plex cytokine Milliplex assay (Millipore). Additionally, the following ELISA kits were used: Human IL-8 ELISA Ready-SET-Go!® (ebioscience), Human TNF ELISA set (BD Biosciences), IL-10 Human PeliPair™ ELISA kit (Sanquin Reagents) and Histamine ELISA kit (Neogen), IgG and IgM ELISA kit (Jackson Immunoresearch).

mRNA sequencing

Total RNA from synovial tissue was extracted using the Qiagen RNeasy mini kit as per manufacturer's protocol including the on-column DNase digestion. Quality control of samples was done to determine RNA quantity and quality prior to their processing by RNA-seq. The concentration of total RNA samples was determined using NanoDrop 8000 (Thermo Scientific). The integrity of RNA samples 1was determined using both 2100 Bioanalyzer and 2200 TapeStation (Agilent Technologies). Where available, 1ug of total RNA was used as an input material for library preparation using TruSeq RNA Sample Preparation Kit v2 (Illumina).

Generated libraries were amplified with 10 cycles of PCR. Size of the libraries was confirmed using 2200 TapeStation and High Sensitivity D1K screen tape (Agilent Technologies) and their concentration was determined by qPCR based method using Library quantification kit (KAPA). The libraries were first multiplexed (five per lane) and then sequenced on Illumina HiSeq2500 (Illumina) to generate 50 million of paired end 75 base pair reads (synovial samples). For the data analysis, the Genomic Short-read Nucleotide Alignment Program (GSNAP) (http://research-pub.gene.com/gmap/) was used to map and assemble transcripts using the UCSC hg19 human genome reference sequence and associated transcriptome map.

RESULTS

Mast cells activation in rheumatoid arthritis

We investigated the activation of mast cells by stimuli known to be present in human synovium and implicated in the pathogenesis of RA, such as IgG immune complexes, specifically ACPA-IgG.

Mast cell activation, evaluated by measurement of CXCL8/IL-8 levels, was observed upon triggering with IgG ACPA immune complexes, which were formed by binding of the serum of patients with ACPA-positive RA to citrullinated peptides (Figure 1, A-B). Importantly, mast cells were not activated by ACPA-positive serum incubated with the arginine control or by serum from ACPA- negative patients. The activation of mast cells by IgG was significantly enhanced by TLR agonists [14], including endogenous ligands (e.g. HSP70), known to be present in synovia (Figure 1, C-D).

Overall, these data indicate that mast cells can contribute to synovial inflammation by producing pro-inflammatory mediators (IL-8, a potent chemoattractant for neutrophils) upon triggering with IgG immune complexes, including ACPA immune complexes and that other stimuli present in synovia (e.g. TLR ligands) can further enhance this response.

This is in line with previous reports indicating a deleterious role for mast cells in RA [52,53] [54], recently reviewed in [30].

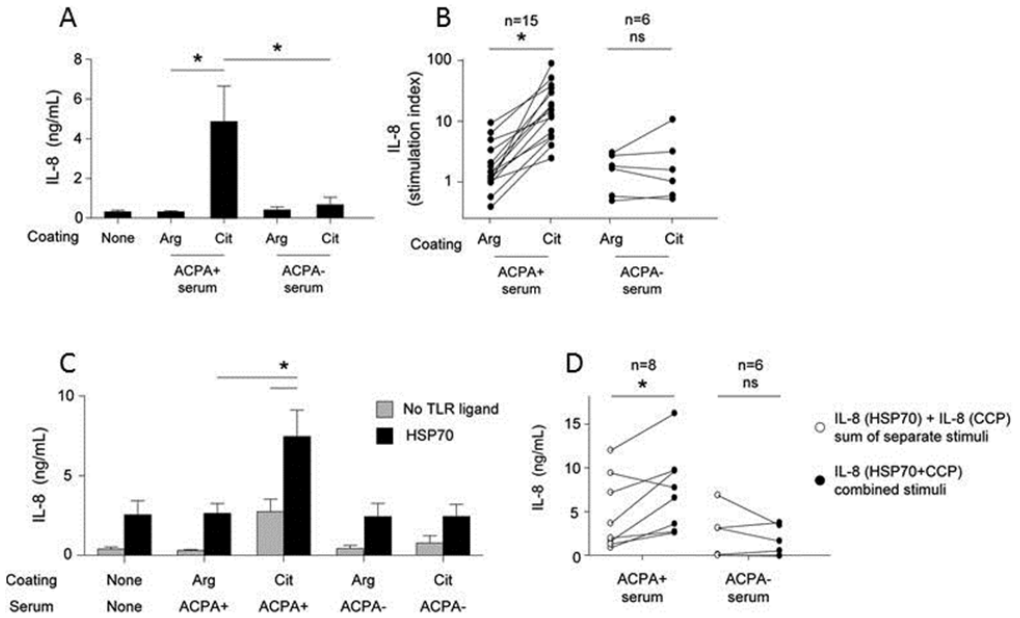

Figure 1. Activation of mast cells by ACPA immune complexes

A, B Mast cells were stimulated on CCP-coated plates incubated with no serum (ctr) or serum of antibodies against citrullinated proteins (ACPA+) and ACPA− rheumatoid arthritis patients, as indicated, for 24 h, after which IL-8 production was measured in supernatant. Results are shown as the summary of all experiments (A) or pairwise (B), with each symbol representing an individual patient serum tested. C, IL-8 production by mast cells in response to combined triggering using plate-bound ACPA and HSP70 or their separate stimulations. Results are shown as the summary of all independent (n=5) experiments. D, IL-8 production after combined stimulation with HSP70 and ACPA (closed symbols) compared to the sum of IL-8 following separate stimulation with HSP70 and ACPA(open symbols). Asterisks indicate significant ($p<0.05$) differences between the indicated conditions, using paired samples t test.

Modified from Suurmond J, Rivellese F, et al. Ann Rheum Dis. 2015 Oct;74(10):1915-23.doi: 10.1136/annrheumdis-2014-205562

Mast cells as immunomodulatory cells in rheumatoid arthritis

Having found that mast cells can be activated by IgG immune complexes, we assessed the influence of IL-33 on IgG-induced mast cell activation. Interestingly, IL-33 was shown to prime mast cells toward a Th2 & immunomodulatory phenotype. In fact, stimulation of mast cells with IL-33 induced higher amounts of histamine and IL-10, compared to the induction CXCL8/IL-8 upon triggering with IgG (Figure 2, A). Likewise, only IL-33, and not pbIgG, induced the upregulation of the mast cell activation marker CD203c (Figure 2, B). The histamine release observed upon IL-33 triggering was not accompanied by the upregulation of CD63, a marker of mast cell degranulation. Combination of the two stimuli led to an increased activation of mast cells, with the release of significantly higher amounts of CXCL8/IL-8, histamine and IL-10, as compared to unstimulated mast cells or to single stimuli (Fig 2 A, last columns). To discriminate between an additive or synergistic effect, we compared the sum of single stimuli with the actual amounts produced upon combined stimulation with IL-33 and pbIgG (Figure 2C, empty Vs filled dots). This comparison showed higher amounts of histamine and IL-10 with combined stimulation, indicating a

clear synergistic effect for these IL-33-triggered mediators, while for CXCL8/IL-8 only an additive effect was observed.

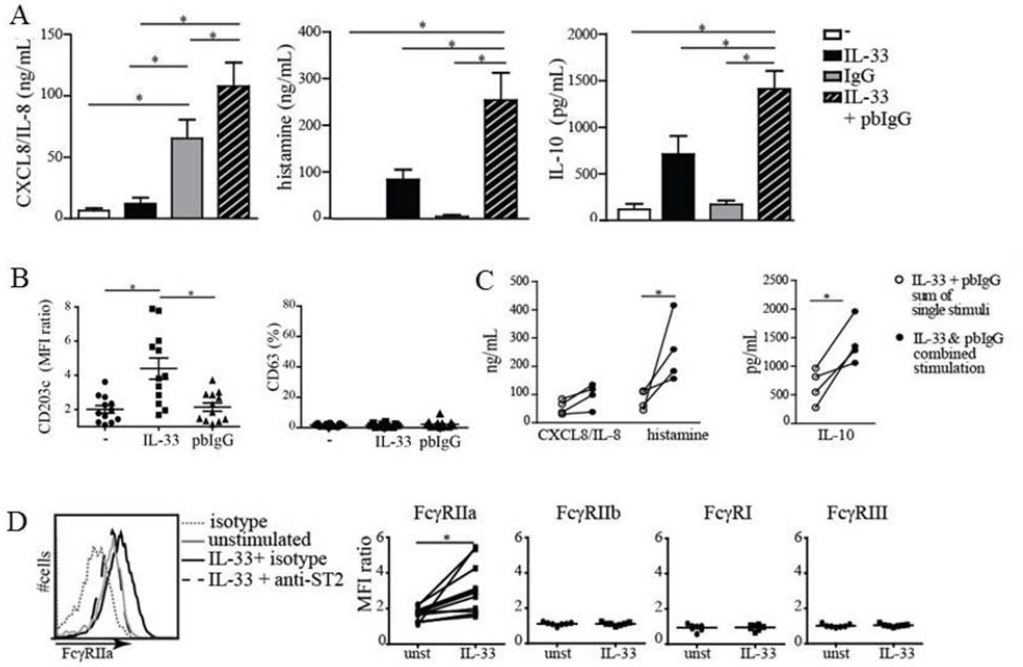

Figure 2. Activation of mast cells via interleukin-33 (IL-33) and immune complexes

Mast cells were triggered with IL-33 or plate bound IgG for 24h. A, CXCL8/IL-8, histamine and IL-10 measured by ELISA. Mean + SEM from 4 independent experiments (n=4). B, Expression of CD203c left, as Median Fluorescence Intensity (MFI) ratio to isotype, and CD63 (right), as % of positive mast cells. Each symbol represent a mast cells donor (n=12) from 5 independent experiments *$p<0.05$ determined by ANOVA with Bonferroni's post-test (for multiple comparisons) C, Synergy was assessed by comparing sums of mediators measured in single stimulations (IL-33 + IgG) and the actual values measured in the supernatants of cells simultaneously stimulated (IL-33 & IgG). Each symbol represents a mast cells donor (n=4) from 4 independent experiments C, CXCL8/IL-8 levels, measured by ELISA, after incubation of mast cells with ACPA+ RA sera bound to citrullinated peptides or arginine controls (n=6 mast cell donors, with sera from 3 ACPA+ patients). D, Histogram (representative of 4 independent experiments, n= 4 mast cell donors) showing FcγRIIa expression by

mast cells upon stimulation with IL-33 (black line) and blocking of IL-33 receptor ST2 (dashed black line). On the right, expression of FcγRs by mast cells without and with IL-33 triggering, as MFI ratio to isotype. Each symbol represent a mast cells donor (n>7) from 5 independent experiments. *p<0.05 determined by ANOVA with Bonferroni's post-test (for multiple comparisons) and by Student t test (for comparisons between two groups). Modified from Rivellese F, et al. Arthritis Rheumatol. 2015 Sep;67(9):2343-53. doi: 10.1002/art.39192.

We next sought to identify the mechanism by which IL-33 enhances mast cell activation and hypothesized that IL-33 modulates the expression of Fcγ receptors on mast cells. Indeed, IL-33 induced a significant up regulation of the activating FcγRIIa (Figure 2 D). This effect was mediated by the IL-33 receptor ST2, as it could be blocked by anti-ST2 antibodies (Figure 2 D). Importantly, we did not detect expression of other FcγRs by cultured human mast cells and these were not influenced by IL33 stimulation (Figure 2 D, right).

Together, these results indicate that IL-33, via its receptor ST2, induces the up regulation of FcγRIIa, enhancing the activation of mast cells upon triggering with pbIgG, as well as ACPA-IgG, as demonstrated by the increased production of CXCL8/IL-8. More specifically, IL-33 induces

IgG-triggered mast cells to release histamine and IL-10, but not CXCL8/IL-8, in a synergistic fashion. To further support the notion that IL-33 is able to modulate mast cell activation by IgG, we performed a multiplex assay on MC supernatants.

In line with previous literature, MCs triggered with IL-33 and pbIgG secreted a wide range of mediators [55-57]. When comparing the two stimulations, we found that pbIgG induced higher amounts of classical pro-inflammatory mediators, while IL-33 induced higher levels of Th2 and immunomodulatory cytokines such as IL-5, IL-10 and IL-13.

Interestingly, as shown in Figure 3A, the combination of IL-33 and pbIgG induced higher levels of these cytokines, when compared to IL-33 single stimulation.

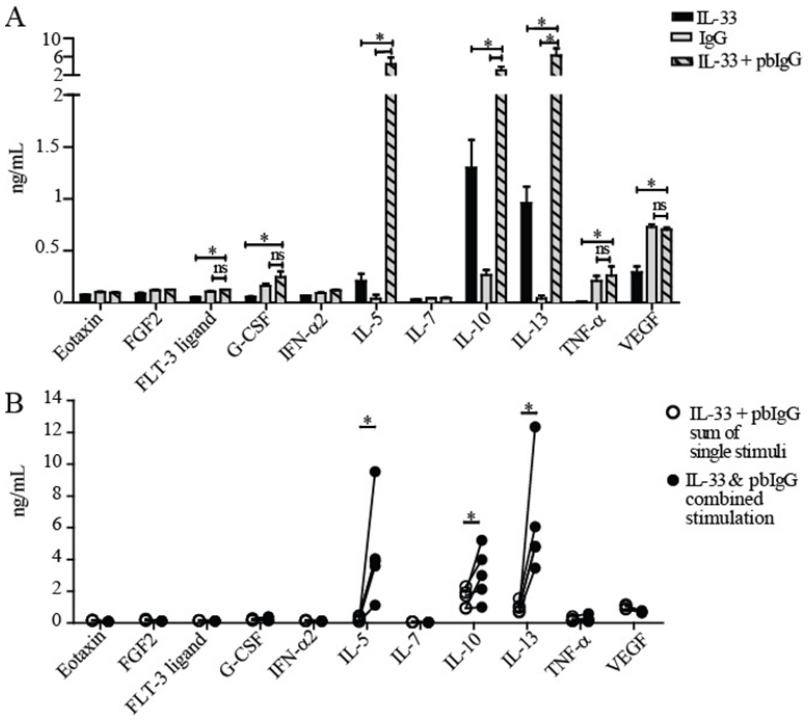

Figure 3. IL-33 skews mast cells toward an immunomodulatory phenotype.

A, comparison between single and combined stimulations with IL-33 and pbIgG. B, Synergy, assessed as in Figure 1B. n=3 mast cell donors, 3 independent experiments, measured by multiplex (only the mediators significantly increased upon stimulation of mast cells with either IL-33 or pbIgG are shown). *$p<0.05$ determined by ANOVA with Bonferroni's post-test (A) and by Student t test (B). Modified from Rivellese F, et al. Arthritis Rheumatol. 2015 Sep;67(9):2343-53. doi: 10.1002/art.39192.

On the contrary, no additional effects of IL-33 could be observed on top of IgG for the pro-inflammatory mediators such as TNF-α or FLT-3Ligand. In line with the data presented in Figure 2B, the combined stimulation showed a synergistic effect that was only present for the mediators induced by IL-33 (Figure 3B).

Altogether, these data indicate that IL-33 is not simply enhancing mast cell activation by IgG, but is actually able to fine tune mast cell activation, inducing the production of a specific set of Th2-associated and immunomodulatory mediators.

Interaction of mast cells with synovial immune cells

To better understand the possible consequences of mast cell activation at the synovial level in RA, we investigated the occurrence of cellular interactions between mast cells and other immune cells *ex vivo*, by performing immunofluorescent stainings for mast cells tryptase together with the monocyte/macrophage marker CD14, the B cell marker CD19 or the T cell marker CD3 on tissue sections from synovia of RA patients. We found numerous tryptase$^+$ cells (mast cells) scattered in the synovium of RA patients. Interestingly, tryptase$^+$ cells showed clear cell-to-cell

interaction with $CD14^+$ (Figure 4A), $CD3^+$ (Figure 4B) and $CD20^+$ cells (Figure 4C).

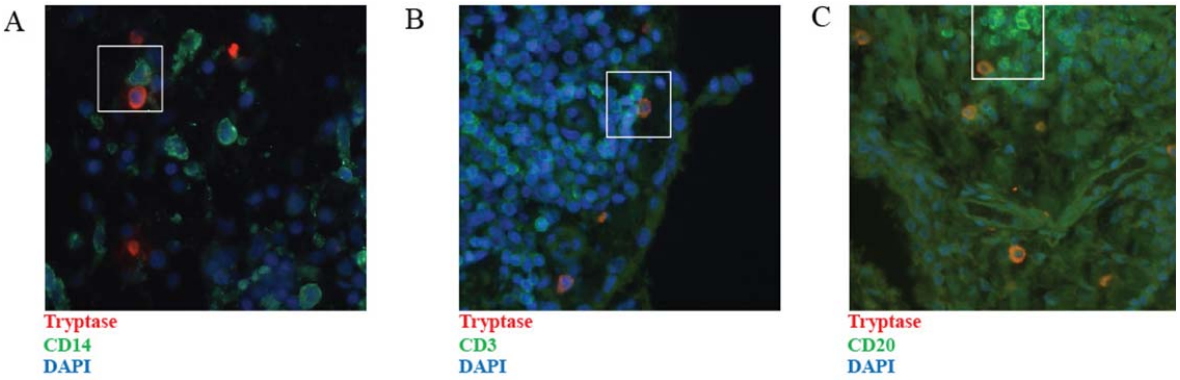

Figure 4. Mast cells interaction with immune cells in RA synovium

A-C, Immunofluorescence analysis of RA synovia, showing tryptase+ mast cells, in red, closely interacting with other immune cells, in green: CD14+ cells (A), CD3+ T cells (B) and CD20+ B cells (C). In blue nuclear staining (DAPI). Representative images from one donor out of three showing similar results. Modified from Rivellese F, et al. Arthritis Rheumatol. 2015 Sep;67(9):2343-53. doi: 10.1002/art.39192.

To quantify these interactions, we counted the number of $tryptase^+$ cells in close contact with each cell type. As shown in Table 1, a substantial proportion of mast cells (mean of 19.5 %) was found to be in contact with $CD14^+$ cells in the synovia of RA patients. Similarly, the proportion of total $CD14^+$ cells interacting with mast cells was considerably higher compared to B and T cells.

These results show that mast cells in the synovia of RA patients are in close contact with immune cells. In particular, a substantial number of synovial mast cells are localized in proximity of synovial $CD14^+$ cells.

Table 1. Interactions between mast cells (MCs) and other immune cells in RA synovia

	RA 1	RA 2	RA 3	Mean*
Mast cells	31.3	11.7	30.7	24.6
Close to $CD14^+$	6.7 (21.4%)	1.7 (14.6%)	6 (19.6%)	4.8 (19.5%)
Mast cells	18.7	24	26.3	23
Close to $CD20^+$	1.7 (9.1%)	3 (12.5%)	0.3 (1.1%)	1.7 (7.3%)
Mast cells	27.3	25.6	38.3	30.4
Close to $CD3^+$	3.3 (12.1%)	1.7 (6.6%)	2 (5.2%)	2.3 (7.6%)
$CD14^+$ cells	80.7	7.0	125.3	71
Close to mast cells	8.3 (10.3%)	0.3 (4.3%)	11.0 (8.8%)	6.5 (9.2%)
$CD20^+$ cells	49.6	45.0	240.3	111.6
Close to mast cells	1 (2.0%)	0.3 (0.7%)	2.3 (1%)	1.2 (1.1%)
$CD3^+$ cells	358.6	0	14.7	124.4
Close to mast cells	6.7 (1.9%)	0 (0 %)	0 (0 %)	2.2 (1.8%)

Means of the number of cells counted in 10 high power fields by 3 independent and blind observers. RA = Rheumatoid arthritis. n=3. *Mean of the three patients.

Modified from Rivellese F, et al. Arthritis Rheumatol. 2015 Sep;67(9):2343-53. doi: 10.1002/art.39192.

Modulation of monocyte responses by mast cells

Since a substantial proportion of mast cells were located near $CD14^+$ cells in synovium (Figure 4 and Table 1), we next examined whether IL-33-primed mast cell supernatants were able to influence the activation of $CD14^+$ cells. To this end, LPS was used to boost the pro-inflammatory activation of $CD14^+$ monocytes, as a model for TLR-4-induced activation of monocyte. Monocyte responses were evaluated in the presence or absence of mast cell supernatants. We used TNF-α production as read-out because mast cells produce little amounts of TNF-α compared to LPS-stimulated monocytes. Figure 5A shows that the supernatants of mast cells inhibited the TLR4-mediated TNF-α production by monocytes. The activation of mast cells with IL-33 and pbIgG significantly enhanced the inhibition of TNF-α production without affecting monocyte survival (measured by DAPI staining, data not shown). This effect was in part dependent on IL-10, as it could be partially inhibited by IL-10 blocking antibodies (Figure 5B). In addition, blocking of histamine receptor 2 with ranitidine also partially reverted TNF-α production by monocytes. When both histamine and IL-10 were blocked, the ability of monocytes to produce

TNF-α was further retained. Importantly, direct effects of IL-33 on monocytes were excluded as no effect on the release of cytokines by monocytes was observed after adding this cytokine to the control media.

Membrane markers of monocyte activation were also evaluated and the influence of mast cell mediators was assessed with blocking experiments. Interestingly, the LPS-induced expression of the co-stimulatory molecule CD80 was reduced by mast cell supernatants (Figure 5C), an effect mainly dependent on histamine, as it could be reverted by histamine receptor 2 antagonist ranitidine.

Altogether, these data indicate that IL-33 triggered mast cells inhibit the pro-inflammatory responses of monocytes, as shown by the suppression of TNF-α production and CD80 expression, presumably through the release of IL-10 and histamine.

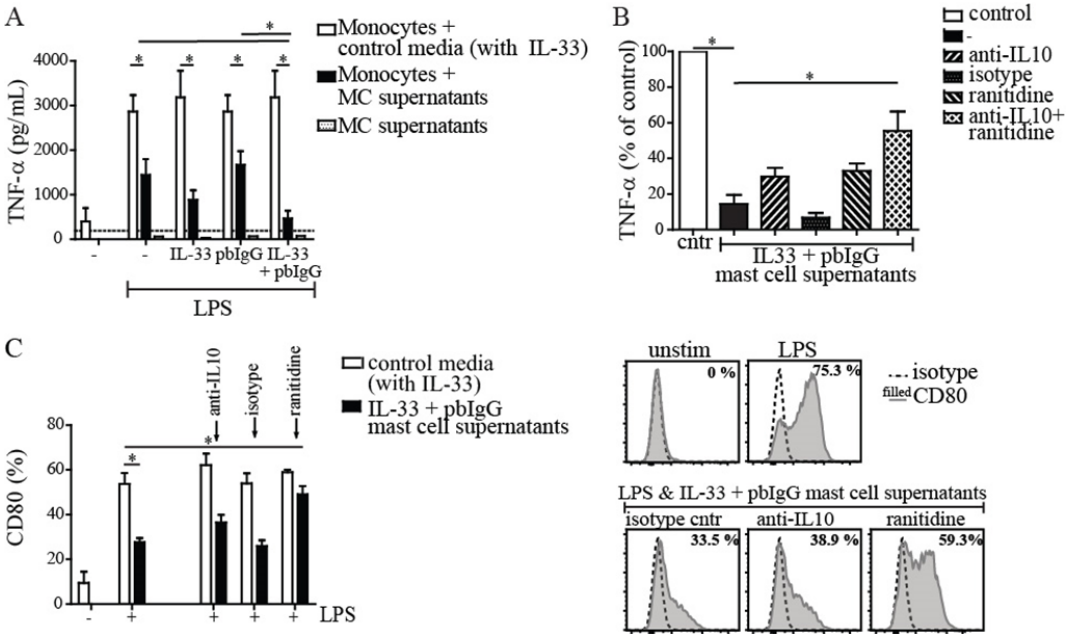

Figure 5. Mast cells modulate monocyte activation

A, Monocytes were incubated with control media, containing the same stimuli used for mast cells (i.e. IL-33, white bars), or mast cell supernatants (black bars) and triggered with LPS. TNF-α levels were measured by ELISA after 18h. TNF-α levels in mast cell supernatants were below detection levels (dotted line). **B,** Monocytes were incubated with the supernatants of activated mast cells and anti-IL-10 blocking antibody, correspondent isotype control and histamine receptor 2 antagonist ranitidine. LPS-induced TNF-α is shown as percentage over control, with control media set as 100%. **C,** Flow cytometry analyses of the expression of CD80 by monocytes triggered with LPS after incubation with IL-33 + pbIgG mast cell supernatants, with IL-10 blocking and ranitidine. A-B, Data is shown as mean + SEM, n=6 monocyte donors and 12 mast cells donors, 5 independent experiments. C, Mean + SEM from n=6 monocyte donors and 8 mast cell donors, 4 independent experiments, with histograms of a representative experiment on the right *$p<0.05$ For comparison between two groups, Student t test was performed. For multiple comparison of conditions, ANOVA was used with Bonferroni's post-test. Modified from Rivellese F, et al. Arthritis Rheumatol. 2015 Sep;67(9):2343-53. doi: 10.1002/art.39192.

Synovial expression of mast cell-related genes inversely associates with disease activity in early RA patients

To investigate whether the mast cell mediated immunomodulatory/homeostatic functions we observed in vitro might have a functional relevance in RA patients, we analysed the mRNA extracted from synovial biopsies of RA patients with early disease (<12 months), naïve to DMARDs therapy. As expected, the levels of mRNA of immune cells markers (e.g. CD3, CD14, CD16) and TNF-α were significantly higher in patients with severe disease activity (i.e. DAS28 >5.2) than in patients with moderate disease activity (i.e. 3.2 >DAS28 ≤5.1) (Figure 6A). In contrast, the opposite observation was made for the mRNA-expression levels of mast cell-specific genes, selected on the basis of a recent study describing highly specific genes for human mast cells (39). Using the mRNA levels of these genes as a proxy for the presence of mast cells, we observed that most of the mast cell-related genes (such as, for example, c-kit, tryptase α and β 1, chymase, etc.) displayed a significantly lower expression in patients with severe disease activity (i.e. DAS28 >5.2) than in patients with moderate disease activity (i.e. 3.2 >DAS28 ≤5.1) (Figure 6B). These data are in line with the observations made in our in vitro studies, as

they suggest that mast cell markers, unlike other immune cells, are not associated with a more severe clinical phenotype.

Finally, the mRNA levels of IL-33 showed an inverse correlation with pro-inflammatory markers, such as CD14, the FcγRIIIa and TNF-α (Figure 6C). Together, these data further support our hypothesis that IL-33 triggered mast cells have an immunomodulatory/homeostatic role in RA and could potentially influence the disease severity in patients with RA.

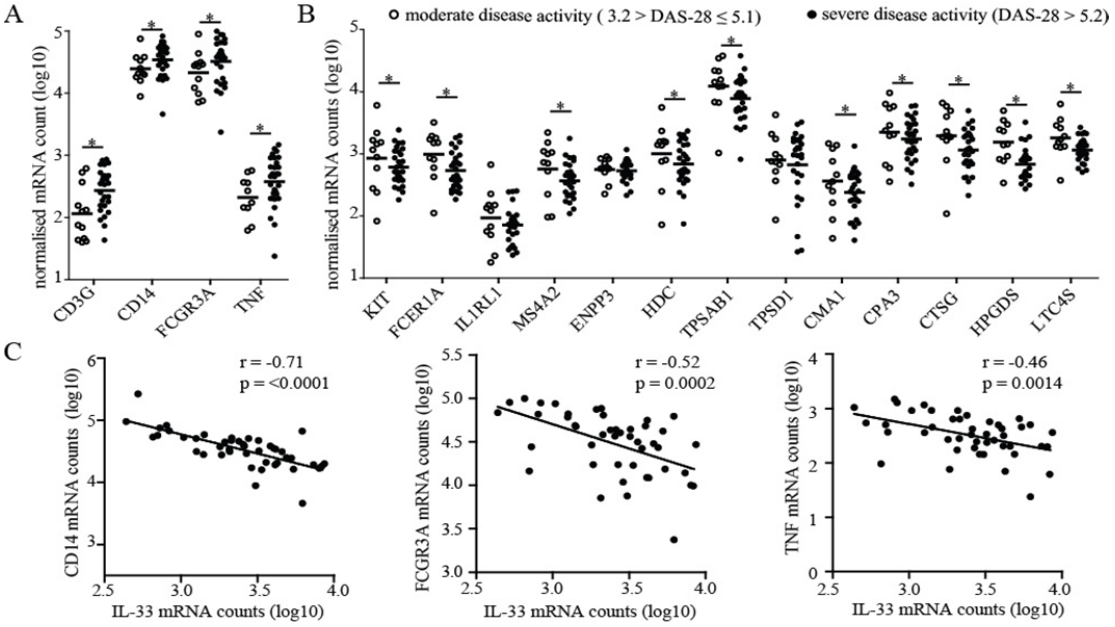

Figure 6. Gene expression in the synovia of early RA patients.

A, mRNA levels of immune cell markers and TNF-α in patients with moderate (3.2 > DAS-28 ≤ 5.1, empty dots) and severe (DAS-28 > 5.2, filled dots) disease activity. B, mRNA levels of mast cell-specific genes in patients grouped as above. C, Correlation of IL-33 mRNA levels with CD14, FcγRIIa and TNF-α. Each dot represents a single patient, with line at the mean in A and B and regression line in C. n=40 *p<0.05 with Student t test performed to compare means of expression levels in two groups of patients

(moderate Vs severe DAS-28) for each gene in A and B and Pearson's correlation in C. Modified from Rivellese F, et al. Arthritis Rheumatol. 2015 Sep;67(9):2343-53. doi: 10.1002/art.39192.

Synovial mast cells correlate with synovial inflammation and cellular infiltration in patient with early RA

Having found that mast cell activation in synovia can be differently induced toward pro-inflammatory or immunomodulatory responses, we wondered if mast cells, as tunable effector cells, could have various functions in different patient subsets, and, possibly, in different disease stages, further adding complexity to the heterogeneity of RA.

To address this question, we analysed the presence of mast cells in the synovia obtained by ultrasound-guided biopsy from a large cohort of patients (n=90) with early RA. Table 2 shows a summary of patient characteristics. Importantly, these patients had disease duration of less than 12 months (early RA) and were naïve to therapy with DMARDs, allowing for the first time the study of synovial inflammation without the biases of long-standing disease and/or immunosuppressive therapy.

Table 2. Summary of patient characteristics (n=90)

Age, years mean (SD), range	52 (16)	19-89
Sex (% Female)	72.3%	
DAS-28 mean (SD), range	5.64 (1.39)	1.88-8.92
ACPA+ %	78.7%	
RF+ %	74.5%	
ESR (mm/h) mean (SD), range	39 (30)	2-120
CRP mean (SD), range	17 (27)	0-162
Disease onset (Months) mean (SD), range	6 (3)	1-12

As shown in Figure 7, MCs were positively correlated with the degree of synovial inflammation, evaluated by the Krenn score, a validated measurement taking into account the enlargement of lining cell layer, the cellular density of synovial stroma and the leukocytic infiltrate [51].

MCs correlated with the infiltration of T cells, B cells, macrophages and plasma cells. Of particular interest is the strong correlation with B cells.

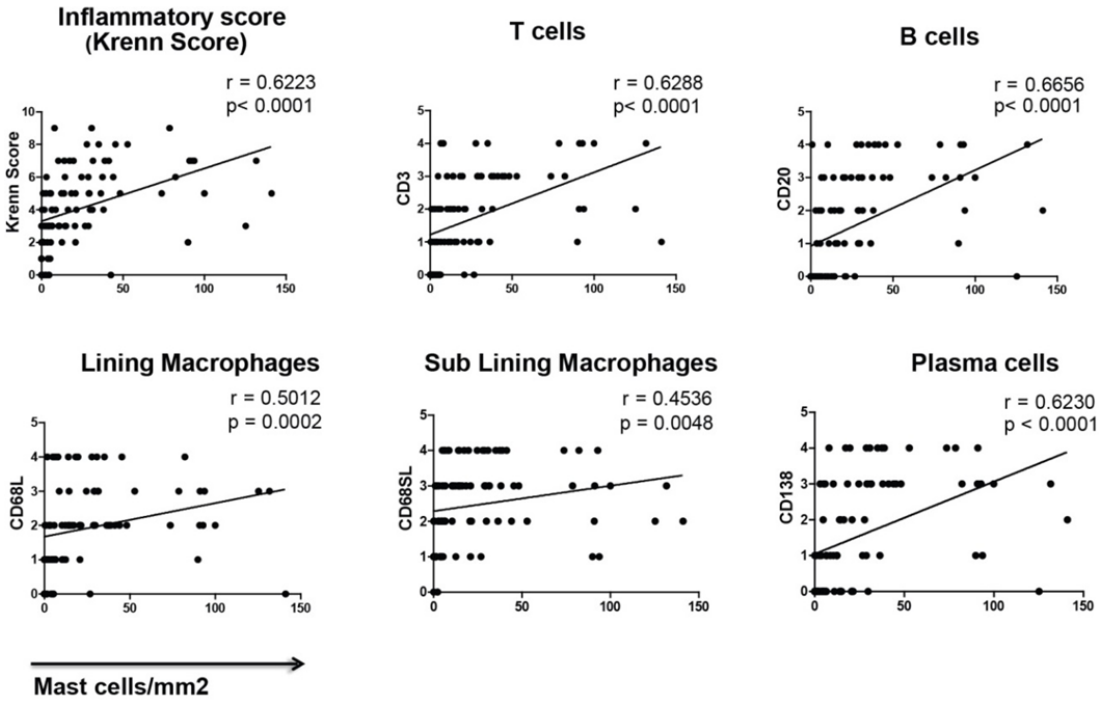

Figure 7. Mast cells correlate with synovial inflammation

Density of mast cells (number of cells/mm2) in relation to synovitis score, and semiquantitative score of T cell, B cells, macrophage and plasma cells. n=90. Spearman correlation.

We have then stratified patients according to MC numbers, as shown in figure 8 A.

The results in table 3 indicate that the groups of patients with high mast cell counts have significantly higher levels of inflammatory markers (CRP and ESR) and a higher prevalence of antibody positivity (both RF and ACPA).

Table 3. Stratification of patients according to mast cell number

		MAST CELLS STRATIFICATION			
	All (n=90, 100%)	**Low (n=29)**	**Medium (n=29)**	**High (n=31)**	**P value***
Female, %	72.3%	68.8%	71.9%	72.7%	0.985
Age, mean (SD)	52 (16)	50 (15)	54 (17)	52 (15)	0.562
ESR, mean (SD)	39 (30)	28 (28)	42 (30)	49 (29)	0.018
CRP, mean (SD)	17 (27)	7 (15)	22 (33)	21 (25)	0.036
RF+, %	74.5%	61.3%	65.6%	93.9%	0.002
ACPA+, %	78.7%	74.2%	68.8%	93.5%	0.042
DAS28, mean (SD)	5.64 (1.39)	5.16 (1.51)	5.85 (1.26)	5.91 (1.31)	0.059
Krenn score, mean (SD)	4.56 (2.17)	3 (1.44)	4.55 (2.14)	5.77 (1.93)	<0.001
Pathotypes					<0.001
Fibroid, %	21.3%	51.6%	12.5%	0.0%	
Myeloid, %	29.8%	35.5%	37.5%	16.1%	
Lymphoid, %	48.9%	12.9%	50.0%	83.9%	

*one way Anova or Chi Square test

Additionally, the level of synovitis, measured by the Krenn score, was significantly different among the three groups, with higher scores in patients with high mast cell counts, in agreement with the previously shown correlations between mast cell numbers and the synovitis score.

Finally, the proportion of lymphoid pathotype was significantly higher in patients with high vs intermediate vs low MC count (83.9% vs 50% vs 12.9%, respectively, $p<0.0001$, table 3 and Figure 8 B). Similarly, synovial MCs were significantly higher in patients with a lymphoid pathotype (mean MC density in lymphoid 41.06/mm2 Vs fibroid/myeloid 11.66/mm2, $p<0.0001$, Figure 8 C).

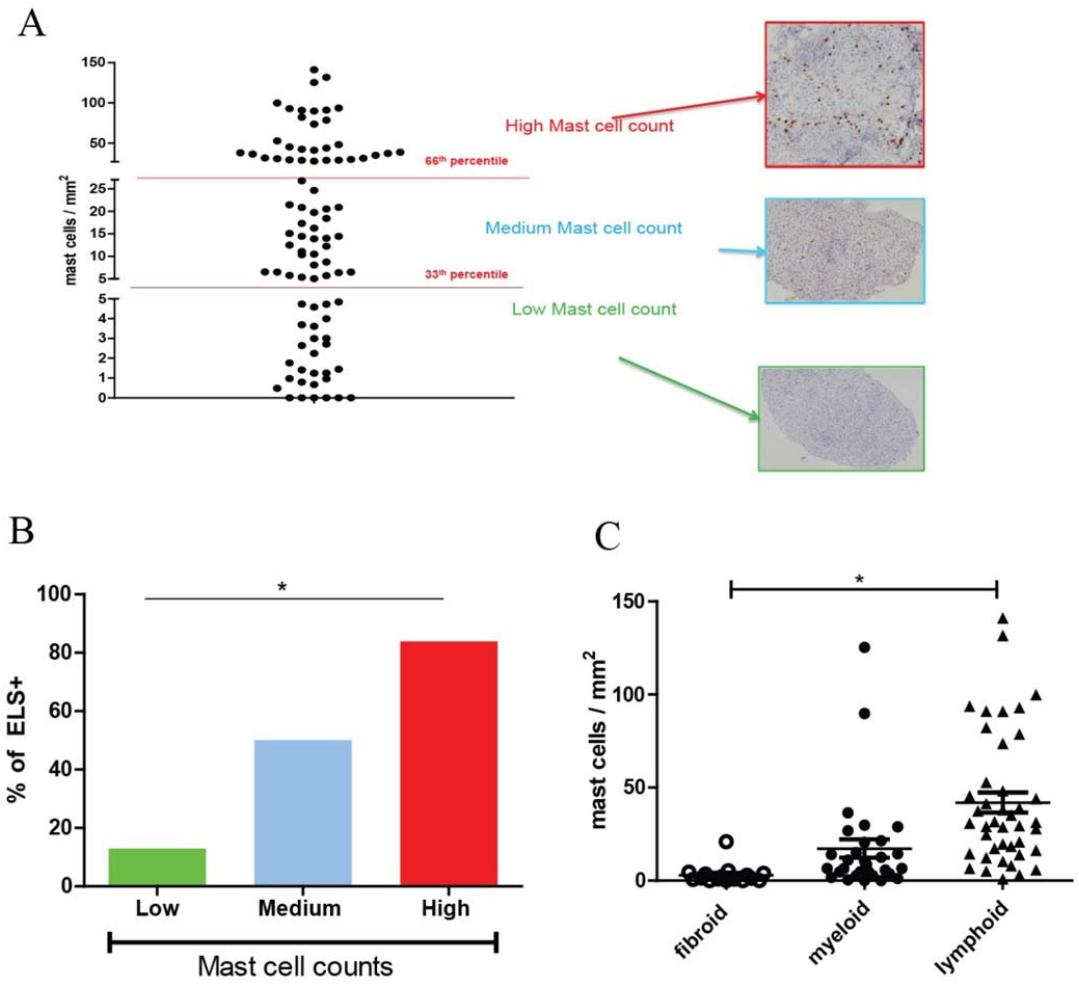

Figure 8. Stratification of RA patients according to mast cell numbers and association with lymphoid pathotype

A, Patients were stratified according to MC numbers as low MC count (<33rd percentile), Medium MC count (33rd-66th percentile) and high MC count (>66th percentile). The pictures on the right show how this stratification, although mathematical, corresponds to a clearly histological difference in the number of infiltrating mast cells. B, % of ELS positivity in patients stratified as in A. C, Density of mast cells in patients stratified according to the synovial pathotype. P<0.05, Chi Square in B and Mann Whitney in C.

Overall, these results indicate that MCs strongly associate with the lymphoid synovial pathotype in early RA patients.

We then studied the cellular interaction at synovial level, using tonsils as a control of an organ with aggregates of B and T cells (i.e. the germinal centres of secondary lymphoid organs). Double immunofluorescence showed MCs bordering the germinal centres in secondary lymphoid organs. Similarly, mast cells were found in close contact with B and T cell aggregates in synovia of RA patients, which is confirming that mast cells have a spatial interaction with synovial B cell aggregates (Figure 9).

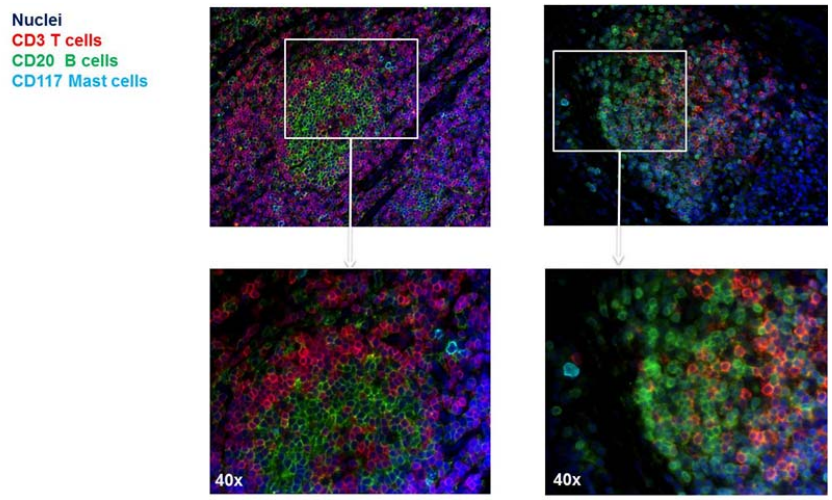

Figure 9. Mast cells bordering lymphoid aggregates in tonsil (left) and RA synovia. Immunofluorescence analysis showing mast cells (in clear blue), T cells (in red) and B cells (in green) in tonsil germinal centres (left) and synovial lymphoid aggregates (right).

Synovial mast cells are functional associated to ectopic lymphoid structures

Having found an association between the presence of mast cells and lymphoid pathotype, and a co-localization of mast cells and lymphoid aggregates in synovia, we wondered whether the interaction between mast cells and B cells has also functional consequences.

To address this hypothesis, we co-cultured primary mast cells with naïve (IgD+) B cells isolated from tonsils. The preliminary results of these experiments suggest that mast cells are able to support B cell survival (Figure 10, A), proliferation (Figure 10, B) and differentiation (Figure 10, C). Interestingly, MCs induced a full differentiation of naïve B cells into antibody producing cells, as demonstrated by and the induction of IgG production upon co-culture of B cells with MCs (Figure 10, D). Importantly, the IgG class-switching was dependent on cell contact, as it was fully abrogated when cells were separated by a transwell membrane. As for the effect of mast cells on survival and proliferation, these were only partially abrogated by transwell, which is suggesting an additional contribution of soluble mediators. Potential candidates are CD40L and IL-6, both known to be expressed/produced by human mast cells, and

additional studies will be needed to unravel the mechanisms underlying the interaction between mast cell and B cells.

Altogether, these data indicate that mast cells are able to support B cell survival, activation and differentiation, suggesting that they may contribute to functional activation of lymphoid aggregates in vivo.

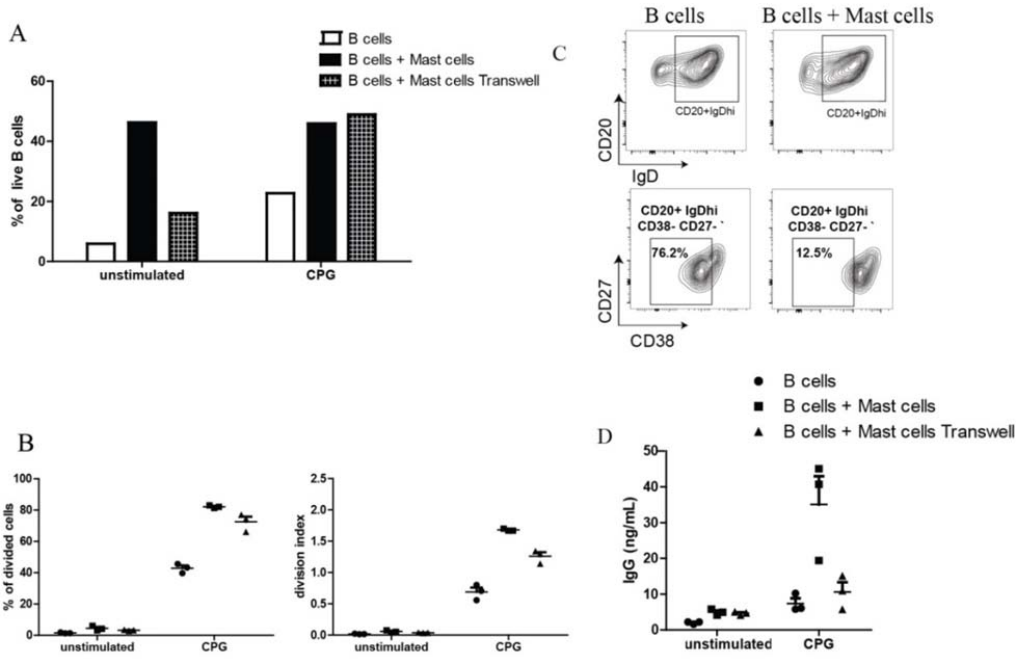

Figure 10 Mast cell and B cell coculture

Naïve (IgD+) B cells isolated from tonsils were co-cultured with primary mast cells. Cells were in contact or separated by a transwell™ membrane. A, proportion of live B cells, in unstimulated cells (left) and cells stimulated with CPG (right). B, Proportion of naïve B cells, gated as CD20+IgDhiCD38-CD27-. C, Proportion of divided cells and division index (average number of cell divisions that a cell in the original population has undergone) calculated by CFSE incorportation. D, IgG measured by ELISA. One preliminary experiment in triplicate.

Synovial mast cells as predictors of radiographic progression

Because our results suggest that high counts of synovial mast cell at baseline are associated with antibody positivity and a higher degree of local and system inflammation, and in particular is with lymphoid-rich inflammation, we hypothesized that the presence of mast cell could identify a subset of patients (a mast cell-rich pathotype) with specific clinical features. As already outlined in table 3, high numbers of mast cells at baseline was associated with higher inflammatory state and antibody positivity, which are typical feature of a severe disease. Accordingly, the number of mast cells showed a positive correlation with inflammatory parameters (ESR and CRP), with disease activity score (DAS28) and its components (tender and swollen joints) (Figure 11).

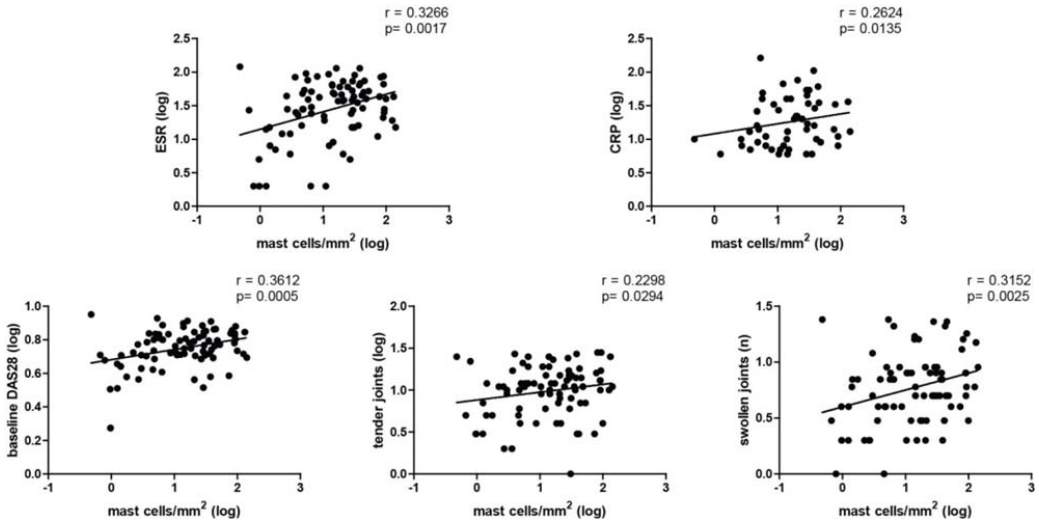

Figure 11. Correlation of mast cell counts with markers of disease activity

This is suggesting that higher number of synovial mast cells is associated with a more severe clinical phenotype. As RA is a chronic disease with a great deal of heterogeneity in terms of progression and response to therapy, we wondered whether the different mast cell counts at baseline would help to predict the disease progression. The PEAC study was an observational study, in which patients were treated according to local guidelines, therefore starting standard therapy according to local and international guidelines with DMARDS and re-assessed at 6 months interval up to 2 years. Most of the patients were treated with DMARD combinations, as specified in table 4.

Table 4. Overview of therapy

DMARDs number	
1	11.80%
2	81.20%
3	7.10%
DMARDs combinations	
MTX	3.5%
HCQ	5.9%
SSZ	2.4%
MTX+SSZ	64.7%
MTX+HCQ	15.3%
SSZ+HCQ	1.2%
MTZ+ASA+HCQ	7.10%

MTX=methotrexate; HCQ=hydroxychloroquine; SSZ=sulfasalazine

Preliminary results from the analysis of the 12m follow-up, shown in table 5, indicate that the baseline presence of mast cells is not associated with differences in inflammatory markers or disease activity at 12 months or with response to therapy, evaluated according to the EULAR criteria [58], or remission rates, defined as a DAS-28 score <2.6.

Table 5. Stratification of patients according to MC counts and 12m follow-up

12m follow-up	All (n=70, 100%)	Baseline MAST CELL STRATIFICATION Low (n=25)	Medium (n=24)	High (n=24)	P value*
ESR	21	14	17	17	0.078
CRP	4	5	7	7	0.216
DAS28	3.73	3.64	3.51	4.19	0.534
EULAR response					0.277
no response	30.6%	42.1%	22.7%	28.9%	
moderate response	25.8%	21.1%	18.2%	38.1%	
good response	43.5%	36.8%	59.1%	33.3%	
DAS28<2.6	37.1%	31.6%	45.5%	33.3%	0.569
Radiographic progression	16.4%	4.0%	12.5%	33.3%	0.018

**one way Anova or Chi Square test.

Interestingly, the proportion of patients showing radiographic progression at 1yr (defined as an increase in the Van der Heijde-modified Sharp score by more than 1 point over 1 year), was significantly higher in patients with high Vs medium and low mast cell counts.

These results suggest that high mast cell counts at baseline are associated with a higher risk of radiographic progression, as shown in Figure 12 A and further confirmed by the analysis in Table 6, showing that the high MC counts (>66th percentile) confer a 12-fold higher risk of RP compared to low MC counts (<33rd percentile).

Table 6. Mast cell stratification and odds ratio of radiographic progression

		Radiographic progression		Total
		no	yes	
Mast cell stratification	<33rd percentile	24	1	25
	>66th percentile	16	8	24
Total		40	9	49
Odds Ratio High Vs Low MC count (95% CI)	12 (1.366 - 105.411)			

This observation suggests that the presence of mast cells in synovia could be used to identify a subgroup of patients with a high risk of radiographic progression.

However, it could be easily objected that mast cells in this context are simply markers of inflammation, as we have previously shown that higher numbers of mast cells are associated with local and systemic inflammation and, more specifically, with lymphoid infiltration. Accordingly, the data in figure 11 B confirms that the lymphoid pathotype is also associated with a higher rate of radiographic progression, which would indicate that the association of mast cells with radiographic progression is indirect. However, as shown in figure 11 C, if patients with lymphoid pathotype are selected, only those with high mast cell counts show high rates of radiographic progression, indicating that mast cell association with radiographic progression is independent from the pathotype.

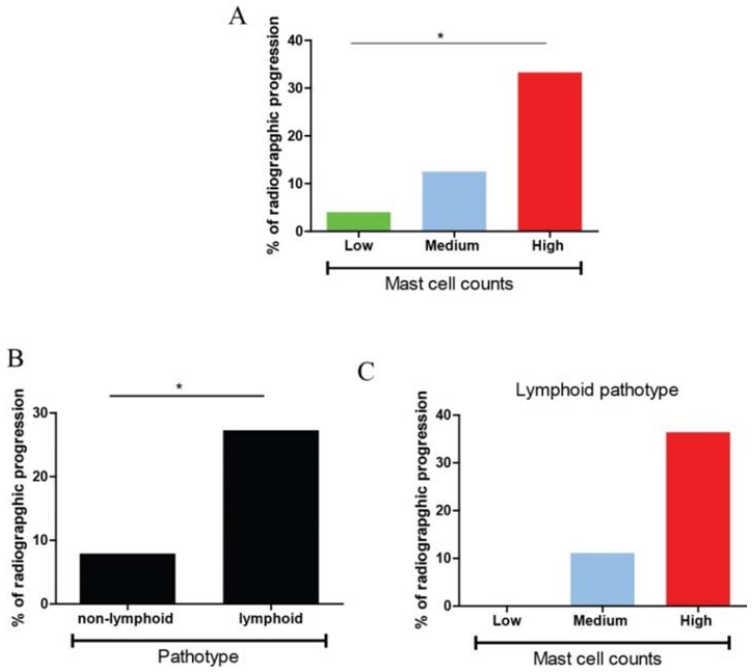

Figure 12. Mast cell counts, pathotypes and radiographic progression.

To identify additional factors potentially associated with radiographic progression, we compared various baseline clinical parameters at baseline in the two groups (radiographic progressors Vs non-progressors). The results of this analysis (not shown), identified the following factors to be significantly different in the two groups: mast cell counts, Krenn score, age at inclusion, presence of ELS and baseline Sharp score. There was no difference in baseline disease activity (DAS-28), inflammatory markers (CRP, ESR) or antibody positivity, which is somehow surprisingly but in line with previous reports showing that single markers, including inflammatory markers, are not always able to predict radiographic

progression[59]. To assess the individual contribution of mast cells to the prediction of radiographic progression, we performed a logistic regression analysis entering the mentioned baseline variables (age, Krenn score, mast cell counts, synovial pathotypes and baseline Sharp score). Performing a backward stepwise regression we found that the best fit, predicting erosions correctly in 86.6% of cases (Nagelkerke R Square of 0.241), could be obtained with a model including mast cell counts (high MC count Vs medium/low MC count), age and baseline Sharp Score (table 7), with mast cells giving the main contribution (p=0.019) to the prediction of radiographic progression. Interestingly, this analysis confirmed that high MC counts confer a significantly higher risk of radiographic progression compared to medium/low MC count (OR 5.7, CI 1.338-24.296). Altogether, these results indicate that mast cells, in addition to being a simple marker of synovial inflammation, could help identifying a specific subset of patients with a higher risk of radiographic progression.

Table 7. Logistic regression analysis of radiographic progression (best fit)

			95% C.I.	
	p	Odds ratio	Lower	Upper
Baseline Sharp Score	.743	.978	.857	1.117
Age	.062	1.049	.998	1.103
MC High VS low/medium	.019	5.702	1.338	24.296

CONCLUSIONS

Mast cells, as part of the inflammatory infiltrate in the synovia of RA patients, exert complex and sometimes controversial effects in the pathogenesis of RA.

In fact, several lines of evidence indicated that mast cell activation can contribute to the unbalanced inflammatory response in the course of RA. Accordingly, we have shown here that mast cells can be activated by immune-complexes formed by ACPA from the sera of RA patients bound to citrullinated antigens, a response that can be augmented by Toll-like receptors triggering.

At the same time, we have also identified IL-33-mediated mast cell activation as a new mechanism able to down-regulate immune responses upon specific triggering in the context of RA. In fact, we found that IL-33 induces human mast cells to release immunomodulatory mediators, such as IL-10 and histamine, as well as other cytokines associated with type 2 immune responses such as IL-5 and IL-13. The release of these IL-33-induced mediators was further enhanced by IgG triggering. In particular, the combined stimulation with IL-33 and IgG showed a synergistic effect that, intriguingly, was present for IL-33-induced mediators (such as IL-5,

IL-10, IL-13 and histamine) and absent for mediators induced by IgG (such as CXCL8/IL-8). These data are important as they indicate that IL-33 is able to prime mast cell toward a Th2/immunomodulatory phenotype, a phenotype that becomes more prominent upon concomitant activation of mast cells by IgG.

Having found such tunable responses by mast cells, we explored their ability to influence the response of other synovial immune cells. Mast cells triggered with IL-33 and pbIgG were able to dampen monocyte activation, inhibiting the production of the prototypical pro-inflammatory cytokine TNF-α and the upregulation of the co-stimulatory molecule CD80. These effects were mediated, at least in part, by the release of IL-10 and histamine from mast cells.

These results indicate that mast cells, in addition to their well-known pro-inflammatory functions, are also able to mediate regulatory/homeostatic responses, in particular when exposed to IL-33.

To confirm the relevance of the latter hypothesis, we studied a cohort of early (<12 months) RA patients naïve to DMARD therapy. Interestingly, while the presence of many types of immune cells, as determined by cell-specific gene-expression, was associated with high disease activity, the

presence of mast cells displayed an inverse association with disease severity. At the same time, IL-33 was inversely correlated with pro-inflammatory markers, such as CD14, CD16 (FcγRIIIA) and TNF-α.

Overall, these data suggest that mast cells can exert both pro- and anti-inflammatory effect, with the net result depending on a fine balance of different triggers. As a consequence, we hypothesised that mast cell contribution to RA could vary in different disease stages and, considering the well-known heterogeneity of RA, they could play different roles in different disease subsets.

To explore this hypothesis, overcoming the discussed limitation of animal models that do not take into account the disease heterogeneity and only study one aspect of the disease process, we directly evaluated the presence of mast cells in synovial tissue obtained by ultrasound-guided synovial biopsies. The analysis of a large cohort of patients (n=90) with early RA, naïve the DMARD therapy, indicated that high mast cell numbers are associated with antibody positivity, with local and systemic inflammation and with the degree of cellular infiltration. In particular, higher numbers of mast cells strongly associate with a lymphoid pathotype. The ability of mast cells to support B cell survival, activation and differentiation in vitro

suggests that they may contribute to functional activation of lymphoid aggregates in vivo, warranting additional studies to establish the role of MC interaction with B cells.

In addition to the implications of these results toward a better understanding of RA pathogenesis, our study also show that synovial mast cell emerge as potential markers for patient stratification. In fact, our preliminary analysis of the 12 months follow-up indicates that the presence of mast cells at baseline represents an independent predictor of radiographic progression at one year. This was independent from the association of mast cells with baseline disease activity and inflammatory markers. Additionally, the association of mast cells with radiographic progression was independent from local inflammation, including the presence of lymphoid-rich inflammatory infiltrate. This observation is of utmost importance, as joint damage is one of the central aspects of rapid progressive disease, and radiographic progression is difficult to predict [60]. For example, high disease activity is considered a negative prognostic factor for radiographic progression, but the exact correlation between inflammatory markers, disease activity and radiographic progression is still being debated[59]. Our results indicate that mast cells are predictors of radiographic progression,

independently from their association with baseline inflammatory markers or disease activity. In other words, within the group of patients with high disease activity, the presence of high numbers of mast cells seem to be specifically associated with radiographic progression. This observation has biological plausibility, as mast cell release several mediators able to induce bone erosions. Accordingly, patients with mastocytosis, a clonal disorder of mast cells, have a deranged bone metabolism with high incidence of osteoporosis and bone fractures[61]. We have also found (preliminary data, not shown) that primary mast cells express RANKL, the most important factor in the regulation of bone metabolism, also involved in bone destruction in RA [62]. However, the exact role of mast cells in the regulation of bone metabolism is unknown and further studies will be needed to unravel the contribution of mast cells to bone erosions in the context of RA. Our observations suggest that the presence of mast cells in synovia could help identifying a subgroup of patients with a high risk of radiographic progression, in which a more aggressive treatment plan should be considered, ideally aimed at preventing bone erosions. In fact, patients stratified according to baseline MC counts did not show any difference at the 12m follow-up in terms of inflammatory markers, disease activity and,

most importantly, response to treatment (table 5). This is particularly interesting considering the recent observation that therapies, mainly with biologicals[63], but possibly also with standard DMARDs [64], may disconnect the relationship between disease activity and joint damage. Accordingly, patients with high baseline MC counts showed significantly higher rates of radiographic progression, which is "disconnected" from the inflammatory markers and disease activity, as these show a similar decrease in all patients at 12 months, independently from their stratification according to mast cell counts. In other words, high mast cell counts seem to identify a group of patients with an aggressive disease. These patients seem to have an apparently good response to therapy, with reduction of inflammatory markers and disease activity score. Nonetheless, in these patients the disease is still active, as they show radiographic progression. Considering that bone damage is the most dreadful consequence of RA, leading to severe disability in the long term, it is clear how these patients would require a more aggressive therapy, ideally aimed at preventing bone erosions, possibly by specifically targeting mast cells.

Our future plans include assessing whether mast cell numbers correlate with response to biological DMARDs, to understand whether mast cells can

further dissect the heterogeneity of RA, allowing the stratification of patients to the right treatment, toward the goal of personalized medicine [65]. Overall, the data presented in this dissertation indicate that mast cells give an important but multifaceted and sometimes controversial contribution to the pathogenesis of RA. The direct analysis of mast cells in the synovia of RA patients is a promising tool to help dissecting the histopathological and clinical heterogeneity of RA. In particular, our data suggest that their presence, independently from local and systemic inflammatory markers and disease activity scores, help identifying a group of patients with high risk of radiographic progression. Additional studies are warranted to better clarify mast cell involvement in the pathogenesis of RA and, from the clinical point of view, assess whether their presence in synovia can help to stratify patients to the most-effective treatment.

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